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X-ray Structure of Yeast Hal2p, a Major Target of Lithium and Sodium Toxicity, and Identification of Framework Interactions Determining Cation Sensitivity

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The product of the yeast *HAL2* gene (Hal2p) is an *in vivo* target of sodium and lithium toxicity and its overexpression improves salt tolerance in yeast and plants. Hal2p is a metabolic phosphatase which catalyses the hydrolysis of 3′-phosphoadenosine-5′-phosphate (PAP) to AMP. It is, the prototype of an evolutionarily conserved family of PAP phosphatases and the engineering of sodium insensitive enzymes of this group may contribute to the generation of salt-tolerant crops. We have solved the crystal structure of Hal2p in complex with magnesium, lithium and the two products of PAP hydrolysis, AMP and Pi, at 1.6 Å resolution. A functional screening of random mutations of the *HAL2* gene in growing yeast generated forms of the enzyme with reduced cation sensitivity. Analysis of these mutants defined a salt bridge (Glu238 ... Arg152) and a hydrophobic bond (Val70 ... Trp293) as important framework interactions determining cation sensitivity. Hal2p belongs to a larger superfamily of lithium-sensitive phosphatases which includes inositol monophosphatase. The hydrophobic interaction mutated in Hal2p is conserved in this superfamily and its disruption in human inositol monophosphatase also resulted in reduced cation sensitivity.

Keywords: inositol; 3′-phosphoadenosine-5′-phosphate; protein crystallography; salt tolerance; site-directed mutagenesis

Introduction

The progressive salinization of irrigated land dictates that an important objective for the agriculture of and regions must be the development of genetically engineered crop plants with greater salt tolerance. Advances in this way will depend on the identification of the primary cellular targets of salt toxicity and elucidation of the underlying mechanisms governing salt responses, which include ion transport, osmotic adjustment and signal transduction (Serrano, 1996). One approach to this complicated problem is the utilization of model organisms, such as *Saccharomyces cerevisae*. A striking discovery in this respect was the identification of a major *in vivo* target of lithium and sodium toxicity, the product of the *HAL2* gene (Glaser *et al.*, 1993; Murguia *et al.*, 1995, 1996). Hal2p is a nucleotidase that hydrolyses 3′-phosphoadenosine-5′-phosphate (PAP) to AMP. It requires magnesium for catalysis and it is inhibited by low concentrations of both lithium and sodium. Inhibition of Hal2p during salt stress results in the accumulation of PAP in the cell, which has the potential to produce a variety of toxic effects such as inhibition of sulphotransferases (Roth *et al.*, 1982) and RNA processing enzymes (Dichtl *et al.*, 1997). In yeast, PAP accumulation also blocks the sulphur assimilation pathway (Murguia *et al.*, 1995, 1996).

As previously observed in yeast (Glaser *et al.*, 1993), the overexpression of *HAL2* in tomato improves salt tolerance (Arrillaga *et al.*, 1998). This demonstrates the applicability of yeast as a model system for the study of the salinity tolerance of

†A. Albert and L. Yenush made equivalent contributions to the work.

Abbreviations used: PAP, 3′-phosphoadenosine-5′-phosphate; IMPase, inositol monophosphatase.

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higher plants. In addition, a family of Hal2p homologues has recently been identified in plants, including three in *Arabidopsis thaliana*, AtAHL, AtSAL1, AtSAL2 and one in rice, OsRHL (Peng & Verma, 1995; Quintero *et al.*, 1996; Gil-Mascarell *et al.*, 1999). These enzymes display varying degrees of cation sensitivity *in vitro* and studies are underway to examine their ability to improve salt tolerance in plant model systems. Therefore Hal2p is the prototype of an evolutionarily conserved family of PAP phosphatases which may be important determinants of salt toxicity. These enzymes belong to a larger superfamily of phosphatases that exhibit an absolute requirement for divalent metal ions and are inhibited by lithium (York *et al.*, 1995). This superfamily includes fructose-1,6-bisphosphate 1-phosphatases (FBPases), inositol polyphosphate 1-phosphatases (IPPases), inositol. monophosphatases (IMPases), PAPases (Hal2p and its homologues) and enzymes acting on both inositol-1,4-bisphosphate and PAP (PIPases) (Lopez-Coronado *et al.*, 1999). Human IMPase has been most extensively characterized and it has been proposed as a target for lithium therapy in the treatment of manic depressive patients (Nahorski *et al.*, 1991).

The elucidation of the atomic structure and the manipulation of cation sensitivity of these sodium-sensitive enzymes have potential applications for improving the tolerance of crop plants to NaCl. Here, we describe the crystal structure of Hal2p complexed with magnesium, lithium, AMP and Pi at 1.6 Å resolution. In addition, as the activity of Hal2p is rate limiting for yeast growth under salt stress, we have identified several residues involved in cation sensitivity using random mutagenesis and *in vivo* selection of salt-resistant clones. Interestingly, the affected residue in one of the Hal2p mutations has a structural equivalent in human IMPase and the analogous mutation confers lithium tolerance to this enzyme.

Results

Crystal structure of Hal2p

The crystal structure of Hal2p (Figure 1(a)) was solved at 1.6 Å by multiple isomorphous replacement techniques using four heavy atom derivatives (Table 1). The enzyme is a monomer of 355 amino acids that can be described as a two-domain structure linked at residue 220. The N-terminal domain is a member of the $\alpha + \beta$ class and the C-terminal domain is α/β with a mixed β-sheet. The mainly antiparallel β-sheet of the N-terminal domain includes β-strands from β1 to β7 and an α subdomain consisting of helices α1 to α4. The C-terminal domain contains an almost parallel β-sheet (strands form β9 to β11), sandwiched between two layers of α-helices. The active site lies between

Table 1. Data collection, structure solution and refinement statistics

Data collection	Native 1	Native 2	Lead acetate	Samarium acetate	K_2PtCl_6	$Gd_2(SO_4)_3$
Space group	$P2_1$					
Unit cell (Å,°)	54.68,	55.44,	55.59,	55.37,	55.36,	54.98,
a,b,c	44.91, 71.60	45.49, 72.35	45.42, 72.46	45.66, 72.41	45.40, 72.13	45.08, 71.98
β	111.08	110.71	111.36	110.70	110.74	111.96
Resolution (Å)	1.6	2.7	3.2	3.0	3.0	3.5
$I/\sigma(I)$ (ove.11.7-1.6 Å)	8.2/1.8					
R_{merge} (%) (ove./1.7-1.6 Å)	7.3/39.1					
Completeness(%) (ove./1.7-1.6 Å)	99.7/100.0					
Multiplicity (ove./1.7-1.6 Å)	5.1/3.7					
Temp. (K)	100	293	293	293	293	293
Number of sites			5	7	6	2
R_{Cullis} (*c/a*)	Reference		0.54/0.51	0.56/0.60	0.68/0.64	0.75/0.84
Phs. power (*c/a*)	Reference		1.55/2.93	1.86/2.40	2.55/1.90	2.35/1.51
R_{Cullis} (*c/a*)	0.30/0.24	Reference	0.62/0.59	0.43/0.40	0.74/0.83	
Phs. power (*c/a*)	4.66/7.40	Reference	1.93/2.04	3.52/4.33	2.20/1.35	
Mean FOM	0.63 (ref.)		0.58 (ref.)			
Refinement						
Resol. range			67.4-1.6			
R_{factor}(overall/1.7-1.6 Å)			19.7/30.2			
R_{free} (overall/1.7-1.6 Å)			23.5/35.1			
Model	354 amino acids, 377 water molecules, one AMP molecule, one PO_4 ion, one SO_4 ion, one beta-mercaptoethanol molecule, 2 magnesium ions					
Ramachandran	Only Ser264 in the generously allowed region					
Electron density	Poor quality at residues 27 to 31; no density for residues 1,356 and 357.					

$R_{Merg} = \Sigma_{hkl}\Sigma i|I_{hkl} - I_{hkl}|/\Sigma_{hkl}I_{hkl}$.
$R_{Cullis} = \Sigma\ [|F_H| - (|F_{PH} - F_P|)]/\Sigma\ |F_H|$ for centric and acentric reflections (*c/a*).
Phasing power = mean value of the heavy atom structure amplitudes divided by the residual lack of closure.

N-terminal and C-terminal domains and is capped by a hairpin or "flap", including residues from 34 to 45 (see Figure 1(a) and (b)).

Crystal structures of inositol monophosphatase (Bone *et al.*, 1992, 1994a,b), inositol polyphosphate 1-phosphatase (York *et al.*, 1994) and fructose-1,6-bisphosphatase (Xue *et al.*, 1994; Villeret *et al.*, 1995) have been reported. These enzymes belong to same superfamily as Hal2p (York *et al.*, 1995) but the low sequence homology (less than 20%) precluded the use of molecular replacement to solve the Hal2p structure. The Hal2p fold is similar to that of these enzymes and the metal sites are structurally conserved at the intersection of topologically equivalent secondary structural elements in a known structures. The main structural differences are in helices α3 and α4, which are not present in other proteins of this superfamily, and in the relative positions of the N-terminal and C-terminal domains.

The crystallization experiments were carried out in the presence of magnesium, lithium and PAP. Two magnesium ions (at sites S1 and S3), a molecule of AMP and a phosphate ion (Pi) can be unequivocally seen in the high resolution electron density map at the active site (Figure 1(c)). Thus, Hal2p has crystallized as a dead end complex with the two reaction products. Mg1 coordinates Glu72 OG1, Asp142 OD1, Ile143 O, a water molecule (W3) and two oxygen atoms of Pi. Mg3 coordinates Glu72 OG2, two oxygen atoms of Pi and three water molecules. Both metal ions are octahedrally coordinated. A third metal binding site (site 2) is occupied by samarium and platinum when crystals are soaked for preparing heavy atom derivatives. This is known to be the lithium binding site in human inositol monophosphatase (Pollack *et al.*, 1993; Wilkie *et al.*, 1995).

Hal2p phosphatase is very sensitive to lithium, presenting a half-maximal inhibition concentration, IC_{50}, of 0.1 mM (Murguia *et al.*, 1995). Hal2p crystals were grown in the presence of 10 mM lithium, so it is likely that lithium is occupying site 2 in the structure although it cannot be seen by X-ray crystallography since the ion has only two electrons. In the native structure, lithium is expected to be coordinated by AMP OH3', Asp294 OD1, Asp142 OD2 and an oxygen of Pi, with distances and angles in agreement with those of lithium-oxygen complexes found in the Cambridge Crystallographic Database (Allen *et al.*, 1991) and also reported by Glusker (1991). The three metal sites are located in an acidic cavity close to the C termini of β1 and β2 from the N-terminal domain and the N-terminus of α8 from the C-terminal domain (see Figure 1(b)).

AMP occupies two pockets in the C-terminal domain (Figure 1(d)). The adenosine ring is located between aromatic rings of Tyr288 and His241. The distance and angle between them, around 3.75 Å and 0 degrees, correspond to those often found in aromatic stacking interactions. The 5' phosphate of AMP occupies a basic pocket, in which Arg281 makes hydrogen bonds to O5, 05'P and the ribose ring oxygen. This arginine is either conserved or replaced by lysine in plant PAP phosphatases (Gil-Mascarell *et al.*, 1999), pointing to an important role for this salt bridge with the 5' phosphate of AMP. His241 ND1, Lys267 NZ and Ser264 main-chain NH are also interacting with O5'P. Ser264 and Lys267 are conserved in plant PAPases (Gil-Mascarell *et al.*, 1999). Several water molecules are also occupying the cavity.

The AMP pocket appears to be holding the nucleotide in a high energy conformation with respect to the ground state (about 6 kcal per mol) (CERIUS2, version 3.0, Molecular Simulations). If the PAP O3' ester substrate adopts the same conformation, with an equatorial disposition of the adenosine ring and 5'PO4 with respect to the ribose ring, it would ensure optimal positioning of the ester by pointing towards the metal binding sites.

Functional screening for Hal2p mutants with altered cation sensitivity

Previous mutagenesis studies with human IMPase have identified several residues affecting lithium sensitivity (Pollack *et al.*, 1993; Gore *et al.*, 1993). Lys36, Glu70, Asp90, Asp93, and Thr95 were shown to be important for catalytic activity, with enzymes mutated at these residues exhibiting less than 0.2% of wild-type activity. In addition, with the exception of Glu70, mutation of these residues decreased the inhibition of the enzyme by lithium and excess magnesium. This was expected because Asp90 and Asp93 form part of metal site 2, that is occupied by lithium, while Glu70 only forms part of metal site 1, a site not involved in lithium or magnesium inhibition. The role of Lys36 must be indirect because this residue does not form part of any metal binding site.

All these mutated residues are conserved in the superfamily of lithium-sensitive phosphatases, corresponding to Lys33, Glu72, Asp142, Asp145 and Thr147 in Hal2p. However, the dual participation of these amino acids in catalysis and metal binding make them of little value for altering the cation sensitivity of active enzymes and therefore for understanding the molecular basis of the differences in cation sensitivity exhibited by different members of the Hal2p and RVIPase enzyme family.

We hypothesized that perturbation of some framework interactions within the enzymes could result in subtle modifications of the active site, preserving catalytic activity but altering sensitivity to inhibitory cations bound at metal site 2. However, it was very difficult to predict from the Hal2p or IMPase structures what kind of interactions determine the cation affinity and specificity of metal site 2. Three residues of human IMPase, His217, Cys218 and Trp219 could be mutagenized to produce active enzymes less sensitive to lithium and magnesium inhibition (Pollack *et al.*, 1993; Gore *et al.*, 1993; Rees-Milton *et al.*, 1997). His217

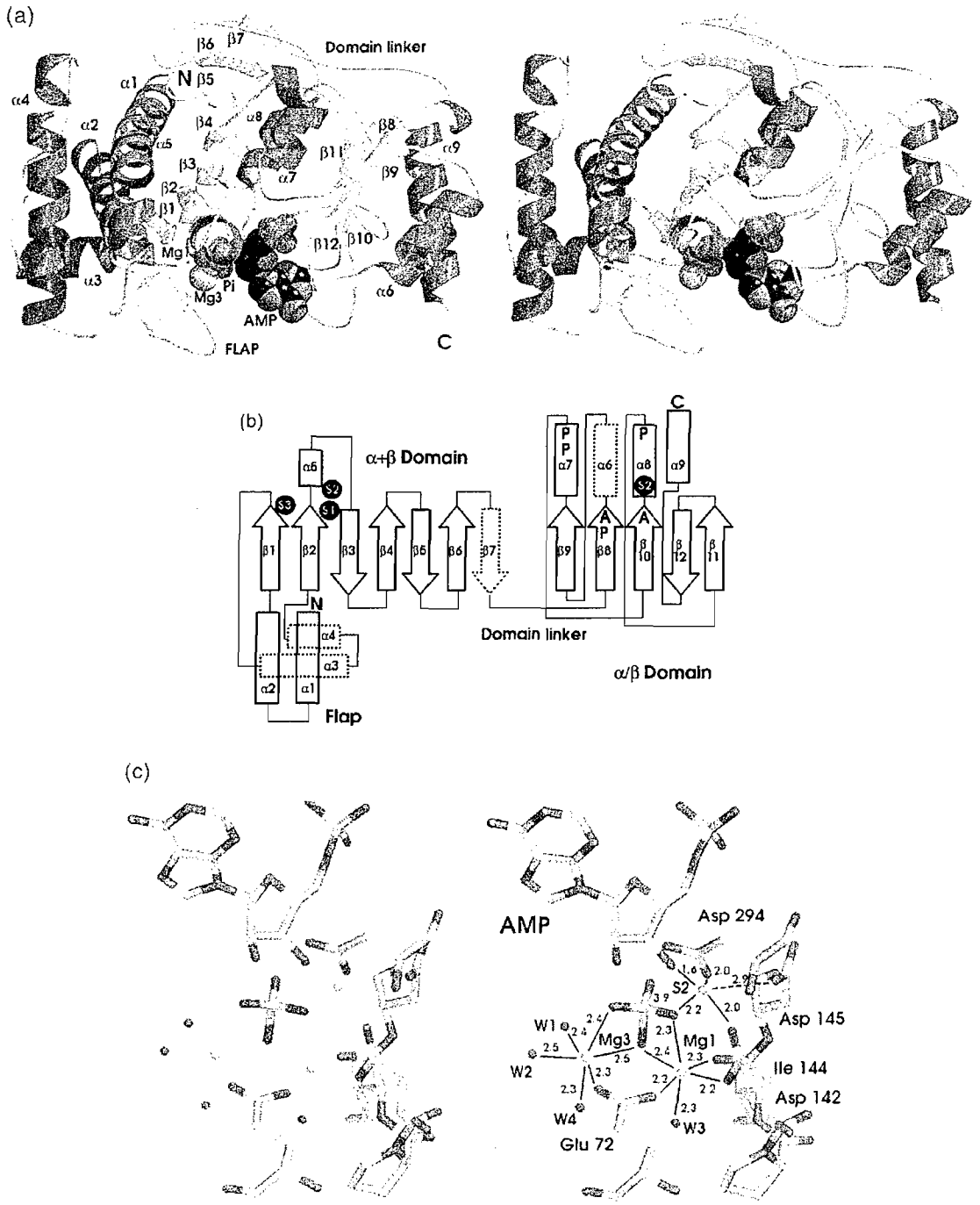

Figure 1 *(legend opposite)*

and Cys218 are not conserved in the family of lithium-sensitive phosphatases and no structural basis for the effects of these mutations were provided.

One important advantage of the yeast Hal2p system is that this enzyme is rate limiting for growth under lithium or sodium stress (Glaser *et al.*, 1993; Murguia *et al.*, 1995). This means that a functional screening can be made *in vivo* for random mutations in Hal2p that result in an active enzyme exhibiting reduced cation sensitivity. As a first step in our analysis of the structural determinants for lithium and sodium inhibition in Hal2p and other phosphatases of the same family, we have generated a library of random mutations in the *HAL2* gene and selected for those enzymes conferring lithium resistance to a yeast strain lacking the native *HAL2* gene. Of the approximately

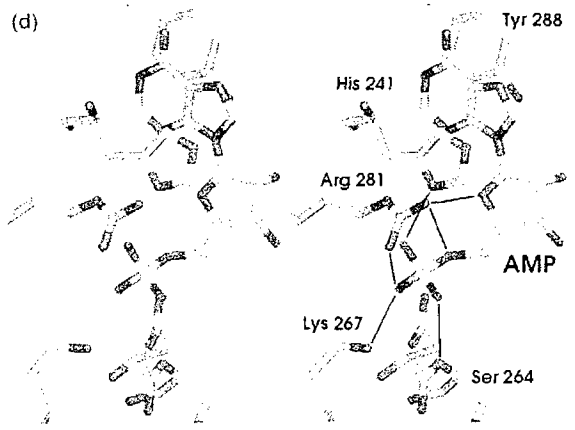

Figure 1. (a) Ribbon representation of Hal2p structure. Magnesium atoms are displayed in gray, AMP and Pi are displayed in CPK mode. (b) Schematic representation of Hal2p showing all the secondary structural elements and binding sites. Continuous lines and broken lines represent conserved or non-conserved secondary structural elements among the sugar enzymes structural class; A is adenosine ring binding site and P is AMP, P5′ binding site. (c) Active site in Hal2p. A view of the metal binding sites (distances are in Å). (d) PAP binding site.

600,000 potential mutations screened, three clones were identified that upon retransformation into yeast improved growth in the presence of 100 mM lithium chloride (Figure 2(a)). Sequence analysis revealed that two of the three clones contained an identical single point mutation resulting in the substitution of Glu238 to Lys (E238K). The third clone contained a distinct point mutation that gives a Val to Ala substitution at amino acid 70 (V70A).

As the PAP-agarose purification protocol used for native Hal2p depends, in part, on the catalytic inhibition by lithium (see Materials and Methods), the genes encoding the wild-type Hal2p and the mutations E238K and V70A were expressed in *Escherichia coli* and purified as histidine-tagged fusion proteins. The *in vitro* characterization of the kinetic properties of the enzymes is summarized in Table 2. The two mutations display increasing levels of lithium and sodium resistance (Figure 2(b) and (c)) while maintaining a relatively high specific activity, as expected from the observation that both proteins complement the absence of Hal2p in yeast (Figure 2(a) and data not shown). As determined by HPLC analysis, the K_m value for the PAP substrate was also altered in the mutant enzymes: the E238 K mutation lowered the affinity for the substrate by fivefold, giving a K_m value of 20 μM as compared to 4 μM for the wild-type Hal2p. By contrast, the V70A mutation displays an even greater substrate affinity. The K_m value for this mutation of Hal2p was below the limits of detection of the BPLC analysis, indicating that the K_m value is less than 1 μM (data not shown).

Construction of an analogous mutation in human inositol monophosphatase

Analysis of the three-dimensional structures of Hal2p and inositol monophosphatases indicated that Val70, but not Glu238, has a structural equivalent in human IMPase (Ile68). We hypothesized that Ile68 of human IMPase, by making an hydrophobic contact with Trp219, may play an analogous role in defining the cation sensitivity of this enzyme. Thus, using a similar approach to that used for Hal2p mutants, we prepared histidine-tagged fusion proteins of both the wild-type and mutant (I6SA) human IMPase. As observed for the V70A mutation in Hal2p, the I68A mutation in human IMPase is much less sensitive to inhibition by lithium chloride (IC_{50} 5 mM, as compared to 0.6 mM for the wild-type), demonstrating that the change in the active site induced by this amino acid substitution (I68A) results in an equivalent change in the lithium sensitivity of human IMPase (Figure 3).

Discussion

The structure of Hal2p described here provides the first characterization at the atomic level of both a PAP phosphatase and a physiological target of sodium toxicity (Glaser *et al.*, 1993; Murgia *et al.*, 1995, 1996; Serrano, 1996; Gil-Mascarell *et al.*, 1999).

The crystal structure of Hal2p is similar to the intermediate state just after hydrolysis of the ester, with the two products of the reaction, AMP and Pi, trapped in a dead-end lithium complex (Figure 1(c)). This is consistent with the fact that, in the presence of lithium, Hal2p binds to the immobilized PAP, thus facilitating the purification and the re-utilization of the affinity column. If this step is performed without lithium, Hal2p extensively hydrolyses the immobilized PAP of the column and affinity purification is much less efficient. These results would support that lithium may act as an uncompetitive inhibitor as in the case of

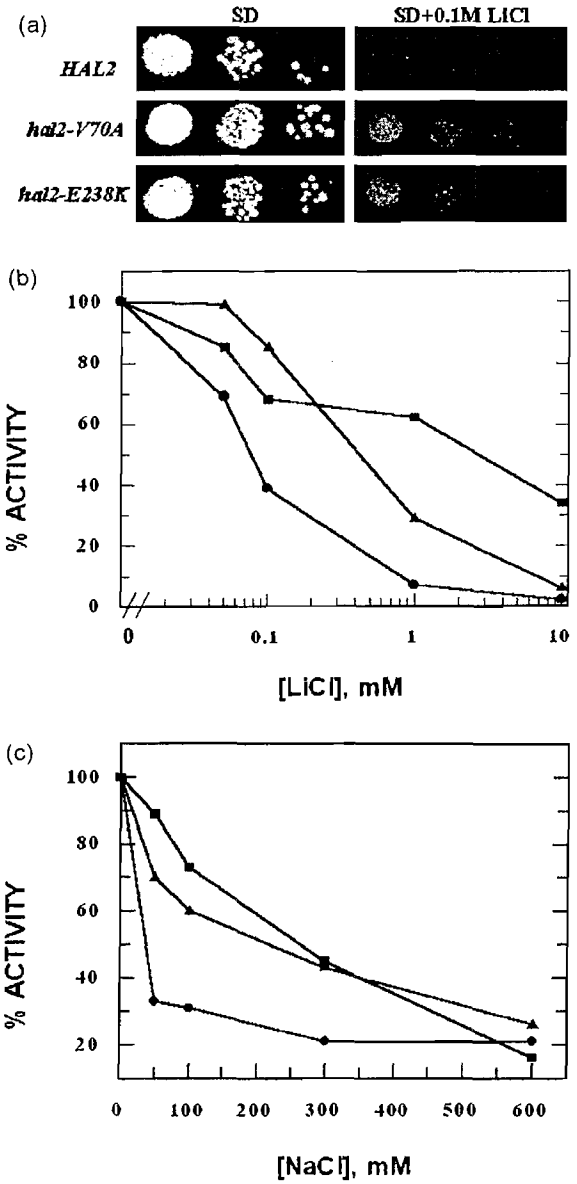

Figure 2. (a) Comparison of the growth of a Hal2- yeast strain expressing the wild-type and mutant *hal2*. Saturated cultures of the three strains were serially diluted and plated on minimal media (SD), with and without lithium chloride (0.1 M). Relative inhibition of phosphatase activity of Hal2p and mutations by lithium chloride (b) and sodium chloride (c). Symbols are as follows: Hal2p (circles); V70A (triangles); E238K (squares). Enzymes were produced as histidine-tagged fusion proteins and incubated with increasing amounts of lithium or sodium. Activity was assayed by the malachite green method, as described in Materials and Methods. Data are expressed as the percentage of activity in the absence of inhibitory cations and are representative of at least two independent experiments performed in duplicate.

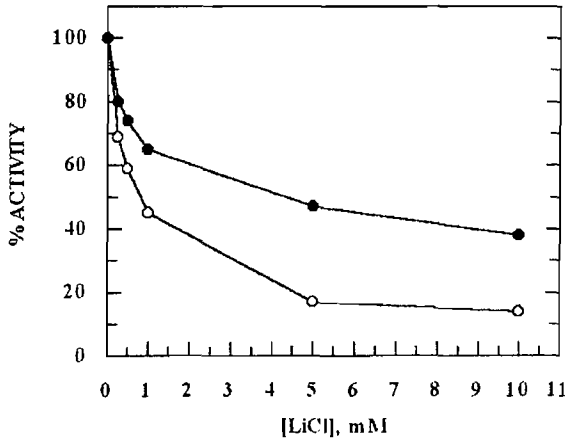

Figure 3. Relative inhibition of phosphatase activity of wild-type (open circles) and mutant (I68A) (filled circles) human IMP by lithium chloride. Experimental procedure is described in Figure 2. Data are representative of two independent experiments performed in duplicate and are expressed as the percentage of activity in the absence of lithium chloride.

other lithium sensitive phosphatases (Nahorski *et al.*, 1991), which means that the inhibition is substrate-enhanced.

We assume that a magnesium ion occupies site 2 and we model Pi bonded to the sugar ring at O3'. Then, Asp294 OD1 and a water molecule would complete the octahedral coordination at this site as in the platinum derivative (see Figure 1(c)). Following Cole *et al.* (1995), in this situation, this water would be close enough to the ester bond to be the nucleophile required for hydrolysis. Once the ester is broken, the leaving Pi would move towards magnesium site 3. The magnesium at site 2 would have fewer ligands and would leave the enzyme together with the AMP and followed by Pi and magnesium site 3. Alternatively, W3 at site 1, would act as the nucleophile (York *et al.*, 1994; Bone *et al.*, 1994a,b; Villeret *et al.*, 1995; Strater *et al.*, 1996). As discussed by Wilkie *et al.* (1995), the final test for these alternative models will come when the stereochemical course of the reaction with respect to the phosphorous atom is determined.

One important difference between the binding of PAP to Hal2p and that of inositol phosphates (Bone *et al.*, 1992, 1994a,b; York *et al.*, 1994) and fructose-1,6-bisphosphate (Xue *et al.*, 1994; Villeret *et al.*, 1995) to their specific enzymes is an aromatic stacking interaction of the adenine ring (Figure 1(d)). Of the two residues involved, Tyr288 is conserved in plant PAP phosphatases, while His241 is conserved in *Arabidopsis* SAL1 but not in the other two members of the Arabidopsis family (SAL2 and AHL). Interestingly, SAL1 has the best affinity for PAP of all the Arabidopsis PAP phosphatases (Gil-Mascarell *et al.*, 1999). Inositol-1,4-bisphosphate resembles the ribose-3',5'-bisphosphate moiety of PAP and it is hydrolysed by

Table 2. *Summary of the kinetic analysis of Hal2p mutants*

	K_m (μM)	K_{cat} (1/seg)	IC_{50}LiCl (mM)	IC_{50}NaCl (mM)
Hal2p	4	1.7	0.07	20
E238 K	20	1.7	4	265
V70A	<1	0.7	0.5	200

Hal2p. However, the K_m of this inositol derivative is very high (about 1 mM), while PAP is hydrolysed with much better affinity (3 μM) (data not shown). The absence of aromatic stacking with inositol-1,4-bisphosphate may explain the poor affinity of Hal2p for this substrate.

Hal2p (Murguia *et al.*, 1995) and other plant PAP phosphatases (SL1, SAL2 and AHL1) (Quintero *et al.*, 1996; Gil-Mascarell *et al.*, 1999) are sensitive to sodium and previous work demonstrates that sodium inhibits in the same way as lithium (Gil-Mascarell *et al.*, 1999). Interestingly, yeast IMPases are sodium-sensitive (Lopez *et al.*, 1999) while animal enzymes acting on inositol phosphates and PAP are sodium insensitive (Lopez-Coronado *et al.*, 1999). It is tempting to speculate that, given the crucial role of these enzymes in cellular physiology, the evolution of animal cells in sodium-containing media (physiological saline) produced sodium-insensitive enzymes. On the other hand, yeast and plants evolved in sodium-poor media and most of their lithium-sensitive phosphatases are also inhibited by sodium. Only in relatively recent times, when the progressive salinization of irrigation land in semi-arid regions has exposed plants to high sodium concentrations, has this "Achilles tendon" of non-animal cells been uncovered (Serrano, 1996).

A detailed comparison of the structure of sodium-resistant animal enzymes with the structure of Hal2p provides some clues for the structural basis of sodium inhibition. A salt bridge between Arg152 and Asp263 in Hal2p forces a displacement of helix α5 (residues from 147 to 152) with respect to its position in sodium-resistant enzymes. In this situation Hal2p Asp145 OD2 is able to make hydrogen bonds to Asp142 OD2, Asp294 OD2 and Gln265 NE1 (Figure 4(a)). These interactions fix the carboxylate side-chain in a conformation that makes the distance from site 2 to Asp145 OD2 larger than in human inositol monophosphatase (Figure 5). There are two direct consequences of this change. First, lithium is no longer coordinated by Asp145; it is replaced in the coordination sphere by O3′ from AMP. Second, this enlargement may enable the formation of a sodium complex that blocks the product AMP in the active site. This salt bridge is not present in the sodium-resistant mammalian enzymes. Arg152 is fully conserved in plant PAPases, while Glu238 is conserved in the most sodium-sensitive of these enzymes, AHL (Gil-Mascarell *et al.*, 1999).

In addition to the structural determinants of sodium-lithium discrimination discussed above, framework interactions affecting inhibition by both lithium and sodium may also be important to engineer sodium-tolerant PAP phosphatases. The characterization of the E238 K mutant reveals an additional role of the salt bridge discussed above in modulating the sodium and lithium sensitivity in Hal2p (Table 2 and Figure 2(b) and (c)). In the wild-type enzyme, the ionic pair between Arg152 and Asp263 is further stabilized by an ionic network involving two adjacent carboxylates Glu154 and Glu238 (Figure 4(b)). It is known that this kind of ion network is essential for increasing the stability and hence, thermoresistance in various enzymes (Goldman, 1995). The mutation E238 K likely changes the stability and the geometry of the salt bridge, the position of α5 and, hence, the sensitivity to lithium and sodium ions. Further evidence of the role of the ionic network can be found by analysing the IC_{50} values for lithium and sodium ions of the PAP phosphatases of *Arabidopsis*. The SAL2 gene product conserves Arg152, Asp263 and Glu154, but Glu238 is replaced by His and thus mimics the E238K mutation of Hal2p. As predicted by the hypothesis, the IC_{50} for both lithium and sodium are very similar for SAL2 and the E238K mutant (10 and 200 mM, respectively). Furthermore, the ionic network is conserved in SAL1 gene product, but Glu154 is replaced by Asp. Despite this conservative change, with respect to wild-type Hal2p, this enzyme is very sensitive to lithium but moderately sensitive to sodium ions (IC_{50} for Li^+ and Na^+ 0.2 and 200 mM, respectively). This phenotype may be explained by a decrease in the physical size of metal binding site 2, which would prevent the larger sodium ions from binding. In the AHL gene product, Asp263 is replaced by Tyr, thus the equivalent salt bridge is not conserved. However, it would be possible to form a salt bridge involving Arg152 and Glu238. This enzyme is highly sensitive to sodium, but not lithium (IC_{50} for Li^+ and Na^+ 10 and 50 mM, respectively (Gil-Mascarell *et al.*, 1999)), possibly suggesting an increase in the size of metal binding site 2, such that the smaller lithium ion is no longer stably coordinated. This natural variability in amino acid sequence and cation sensitivity would imply that small changes in the size and/or geometry of site 2, as defined in part by this ionic network, modulate the cation affinity of this site.

Additional structural elements determining cation sensitivity have been revealed by the V70A mutation. This mutation is also resistant to both sodium and lithium inhibition, but the structural mechanism of this change in sensitivity is difficult

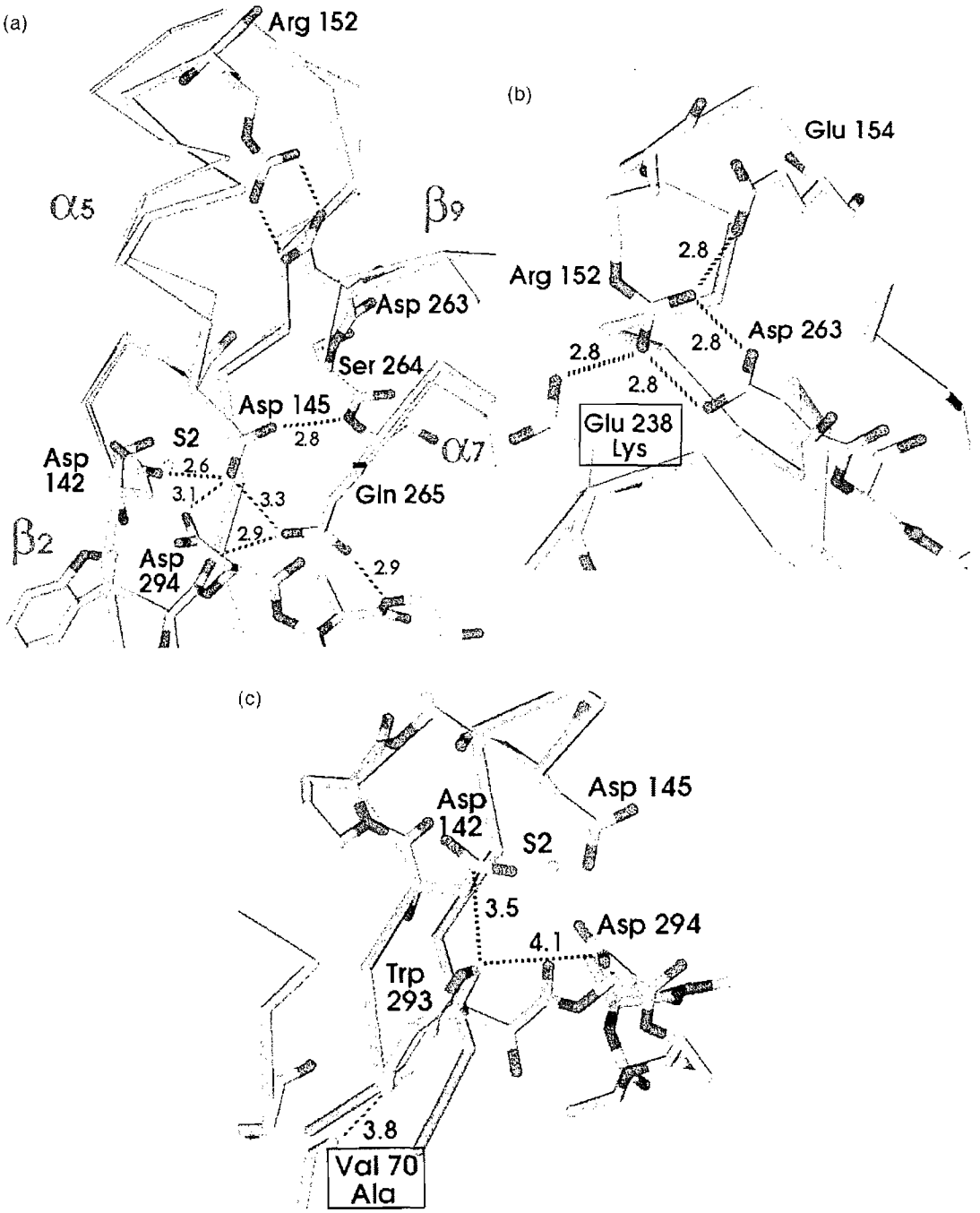

Figure 4. (a) A diagram showing the superposition of Hal2p (thick yellow C^{α} trace) and human inositol monophosphatase (thin cyan C^{α} trace); the Figure shows the hydrogen bonding network involving Asp145 and the salt bridge Arg152 Asp263, linking α5 and the loop between β9 and α7. (b) A detail of this salt bridge and (c) a section of Hal2p structure showing where the point mutation V70A is located. Point mutations are highlighted, distances are in Å.

to predict based on the crystallographic structure information (Table 2 and Figure 2(b) and (c)). Val70 is strictly conserved among Hal2p and the PAP phosphatases of *Arabidopsis* and it is located between the N and C-terminal domains. Val70 CG1 is making hydrophobic interactions with essential Trp293, which holds the carboxylates Asp142 and Asp294 at site 2 (Figure 4(c)). These carboxylates are directly involved in metal binding so, a subtle modification in their conformations would result in an altered specificity at this site, as is observed in the biochemical analysis. Another possible explanation is that this mutation affects the relative orientation of the N and C-terminal domains. This type of change is likely to affect the geometry of the metal binding sites of the enzyme

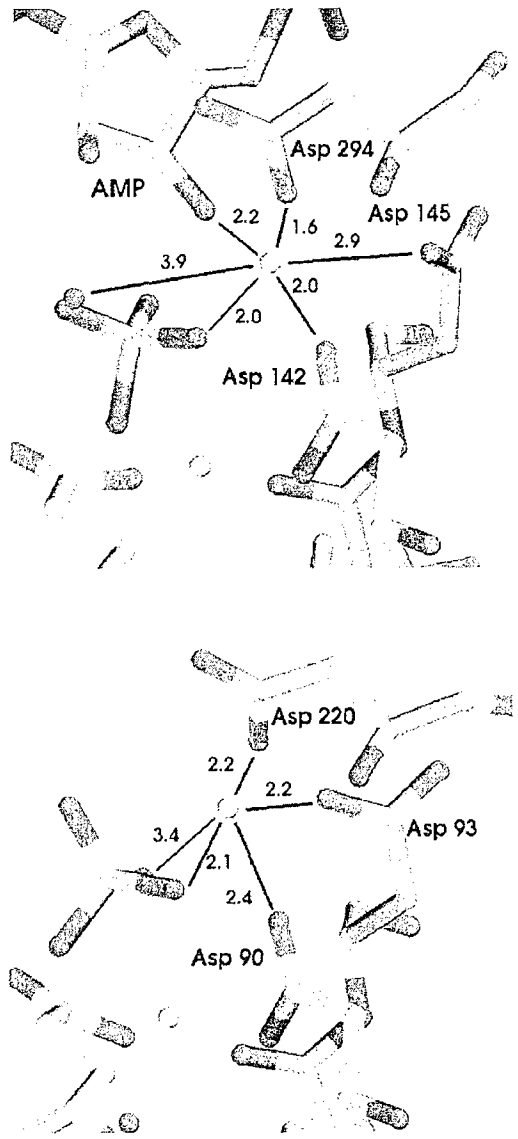

Figure 5. Comparison of metal binding site 2 in Hal2p (top) and in human inositol monophosphatase (bottom). Distances are in Å.

since they are located at the interface between the two domains. This question could be answered by solving the crystal structure of this mutation and comparing the resulting changes in the architecture of the active site.

Given the similarity in the structures, we hypothesized that the information learned for Hal2p may be applicable to human IMPase. Analysis of the superimposed structures indicates that Glu238 of Hal2p has no structural analogue in human but that Val70 is equivalent to Ile68 of human IMPase. As predicted, substitution of Ile68 for alanine renders human IMP approximately tenfold more resistant to inhibition by lithium chloride *in vitro* (Figure 3). This phenotype is similar to that observed for the V70A mutation of Hal2p and demonstrates that the structural change induced by this amino acid substitution results in a similar modification in the binding characteristics of metal binding site 2 in both enzymes. By analogy to the situation in Hal2p, the effect of the I68A mutation must be mediated by the altered hydrophobic interaction with Trp219 affecting the position of active site Asp220. It is plausible that the three previously described mutations of HAPase affecting cation sensitivity (H217Q, C218A and W219F) (Gore *et al.*, 1993; Rees-Milton *et al.*, 1997) also act by displacing Asp220.

As a general conclusion we propose that the combination of structural and mutagenic analysis of Hal2p started here may contribute to the generation of salt-tolerant crops by the genetic engineering of sodium-tolerant PAP phosphatases.

Materials and Methods

Yeast strains and vectors

The *S. cerevisiae* strain used here was RS1051 (*MATa leu2-3,112 ura3-251,328,372 hal2::URA3*) (Glaser *et al.*, 1993). For crystallographic studies, *HAL2* was subcloned into the yeast multicopy vector pRS421, which carries a *LEU2* marker. This vector is derived from Yep 351 (Hill *et al.*, 1986) where an expression cassette, containing the promoter (0.75 kb) and terminator (1.1 kb) regions of the yeast *PMA1* gene, has been introduced into the *Hin*dIII site. For the mutant screening, *HAL2* was subcloned into a centromeric, *LEU2*-containing, shuttling vector, pUN100 (Ellege & Davis, 1988).

Hal2p purification from yeast

For crystallographic studies, native Hal2p was purified by affinity chromatography using a PAP-agarose matrix (adenosine 3′5′-diphosphate agarose, SIGMA, ref. A3640). This method is based on that of Ramaswamy & Jakoby (1987). The crude extract from strain RS1051[pRS-421-*HAL2*] was prepared by resuspending collected cells in a buffer containing 1.2% (w/v) sucrose, 10 mM Tris-HCl (pH 7.6), 20 mM KCl, 1 mM EDTA, 0.2 mM DTT, 0.5 mM PMSF, and 25 mg ml^{-1} of chimostatin. Following physical disruption of the cells, the soluble extract was made 1 mM with respect to $MgCl_2$ and 6 mM with respect to LiCl and then loaded onto the column. The column was washed with buffer 1 (1 mM $MgCl_2$, 6 mM LiCl, 20 mM Tris-HCl (pH 7.6), 5 mM 2-mercaptoethanol, 20% sucrose) and Hal2p eluted with buffer 2 (10 mM EDTA, 20 mM Tris-HC1(pH 7.6), 5 mM 2-mercaptoethanol, 20% sucrose). Hal2p was purified to homogeneity with an overall recovery of 1% (1 litre yeast culture gave 3 mg).

Functional screening for lithium tolerant hal2 mutants

A library of random *HAL2* mutants was generated by introducing the pUN100-*HAL2* vector into the *E. coli* XLI-Red strain (*mutD*$^-$; *mutS*$^-$; *mutT*$^-$) and preparing DNA as recommended by the manufacturer (Stratagene). The yeast strain RS1051 was transformed with the *HAL2* random library and transformants were initially selected

by leucine prototrophy in minimal medium (0.7% (w/v) yeast nitrogen base, 2% (w/v) glucose and 50 mM succinic acid, pH 5) supplemented with methionine (100 μg ml^{-1}). Approximately 1.5×10^5 primary transformants were obtained and pooled together. In order to identify clones which both complement the methionine auxotrophy of the original *hal2* strain and exhibit improved lithium tolerance, the primary transformants were spread on minimal media lacking methionine and containing 100 mM lithium chloride. After secondary screening of 63 possible mutants, ten clones were subjected to plasmid rescue and phenotype confirmation upon retransformation into the original *hal2* strain.

Generation of site-directed mutants of inositol monophosphatase

Isoleucine 68 of human inositol monophosphatase (McAllister *et al.*, 1992) was changed to alanine using an overlapping PCR approach. The primers used were as follows: 5′ external: 5′-ATTATA-**GAGCTC**ATGGCTGA TCCTTGGCAGGAATGC-3′; 3′ external: 5′-CG**CTCG AG**CGGTTAATCTTCGT-3′; 5′ internal: 5′-AAGTATC-CATCTCACAGTTTCGCTGGTGAA-3′; 3′ internal: 5′-AGCTGCCACAGATTCTTCACCAGCGAAACT-3′. The product of the second round PCR was digested with *Sac*I and *Xho*I and subcloned into the pET28a vector (Novagen) to produce a histidine-tagged fusion protein. All constructs were confirmed by sequencing. (Restriction sites are in bold face, the start and stop codons are underlined, and the mutated base-pairs appear in italics.)

Expression of Hal2p and mutants in *E coli* and protein purification

As the purification procedure of native Hal2p by PAP-agarose chromatography depends on inhibiting the enzymatic reaction with lithium, we purified the lithium-resistant *HAL2* mutations as poly-histidine tagged fusion proteins. The open reading frames of Hal2p, human inositol monophosphatase and the mutant forms were amplified by PCR and subcloned into the *Sac*I and *Hin*dII sites of the pET28a vector (Novagen). All constructs were confirmed by sequencing and the proteins were produced and purified from the *E. coli* strain BL21(DE3) using metal chelation chromatography, as recommended by the manufacturer. The proteins were visualized and quantified using SDS-PAGE.

Phosphatase enzymatic assay

Phosphatase activity was assayed by quantifying the inorganic phosphate liberated in each reaction using the malachite green procedure (Baykov *et al.*, 1988) as previously described by Murguia *et al.* (1995).

Crystallization, data collection, structure solution and refinement

Crystallization experiments were carried out in the presence of the natural substrate and the metal cofactors required for catalysis and inhibition. Protein was concentrated to 5 mg ml^{-1} and made 5 mM $MgCl_2$, 0.6 mM PAP and 5 mM Li_2SO_4. Experiments were carried out at room temperature. Crystals were grown by vapour diffusion from drops containing Hal2p and reservoir (30% (w/v) PEG5000 MME, 0.1 M sodium acetate, 5 mM beta-mercaptoethanol, 0.1 M Mes pH 6.5) solutions in a 1:1 ratio. X-ray data from native and derivatives were collected using CuK_α radiation on an in-house source at room temperature (Mar 345 and R axis 4 image plate detectors on Enraf Nonius and Rigaku rotating anode generators, respectively). Native crystals were also frozen to collect a higher resolution data set to be used for refinement. The data were processed using MOSFLM (Leslie, 1987) in space group $P2_1$. The coordinates of one site from the lead acetate derivative were determined using the Patterson interpretation routine of SHELX (Sheldrick, 1991). All the other sites were obtained by difference Fourier techniques and by cross-phasing with the known lead phases. All calculations were performed using programs of the CCP4 package (Bailey, 1994) and the final refinement and solvent flattering were performed using SHARP (de la Fortelle & Bricogne, 1997). Despite the good phasing statistics after refinement of the heavy atom positions, some areas of the solvent flattened map were uninterpretable. This was due to the similar positions of lead, platinum, gadolinium and samariurn heavy atoms. All were bound to the magnesium sites. This caused an overestimation in the figures of merit and an underestimation of the phase error. Following Chabriere *et al.* (1999) we used the lead derivative as native and the native as one derivative. This improved the scaling process, so that it was easier to find minor heavy atom positions, and estimate the phase errors. In this way the phasing statistics were slightly worse for the derivatives sharing heavy atom sites but probably more meaningful. Using both the conventional experimental and the map calculated following Chabriere *et al.* (1999), we were able to build a model, using O (Jones *et al.*, 1991) and refine it using the torsion dynamics routine of XPLOR (Brunger, 1988) at 2.5 Å resolution. Several cycles of restrained refinement at high resolution using REFMAC (Murshudov *et al.*, 1997), followed by interactive model building were carried out (see Table 1). The stereochemistry of the model was verified with PROCHECK (Laskowski *et al.*, 1993). Ribbon figures were produced using MOLSCRIPT (Kraulis, 1991) and RASTER (Merrit & Murphy, 1994). Energy minimization of the AMP molecule was performed using CERIUS2 default force fields and excluding those terms corresponding to bond distances and angles.

PDB accession codes

The coordinates and structure factor amplitudes are deposited in the Protein Data Bank (PDB code: 1QGX).

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